Meningococcal inner membrane proteins and their role in transformation

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Oslo, March 2009

Emma Lång

Abbreviations

BBB	Blood-brain barrier
BFP	Bundle-forming pili
bp	Base pair
CNS	Central nervous system
CSF	Cerebrospinal fluid
DUS	DNA uptake sequence
EMSA	Electromobility shift analysis
Gc	Gonococci
GGI	Gonococcal genetic island
GSP	General secretory pathway
HGT	Horizontal gene transfer
HTH	Helix-turn-helix
IM	Inner membrane
LOS	Lipo-oligosaccharide
LPS	Lipopolysaccharide
Mc	Meningococci
MS	Mass spectrometry
OM	Outer membrane
OMP	Outer membrane protein
OMV	Outer membrane vesicle
ORF	Open reading frame
TLR4	Toll-like receptor 4
3R	DNA repair, recombination and replication

List of publications

I **Emma Lång**, Kristine Haugen, Burkhard Fleckenstein, Håvard Homberset, Stephan A. Frye, Ole Herman Ambur, and Tone Tønjum.
Identification of neisserial DNA binding components.
Microbiology. 2009 Mar;155(Pt 3):852-862.

II Afsaneh V. Benam, **Emma Lång**, Kristian Alfsnes, Håvard Homberset, Burkhard Fleckenstein, Alexander D. Rowe, Ole Herman Ambur, Stephan A. Frye, and Tone Tønjum.
The competence lipoprotein ComL directly interacts with DNA.
Manuscript in preparation.

III **Emma Lång,** Seetha Balasingham, Håvard Homberset, Alexander D. Rowe, Burkhard Fleckenstein, Ole Herman Ambur, Stephan A. Frye, and Tone Tønjum.
The neisserial inner membrane protein PilG directly interacts with DNA and pilus biogenesis components in transformation.
Manuscript in preparation.

The papers will be referred to by their Roman numerals.

1. Introduction

1.1 The pathogenic Neisseria

Neisseriaceae is a family of β-proteobacteria which contains a variety of bacterial genera including the genus *Neisseria*. *Neisseria* species are Gram-negative cocci, which normally adhere to each other, forming so-called diplococci. Many neisserial species are mammalian commensals or part of the normal flora, while two species are major pathogens, *Neisseria meningitidis* and *Neisseria gonorrhoeae*.

N. meningitidis (the meningococcus, Mc) is a human-specific pathogen that colonizes the upper airways, more precisely the oro- and nasopharyngeal mucosal surfaces. Mc may be considered as part of the normal flora and asymptomatic carriage is common. In the general population, around 10% is colonized by Mc at any time, while the colonization rate can be up to 40% in crowded environments such as childcare facilities and military camps (Cartwright *et al.*, 1987; Yazdankhah & Caugant, 2004). Occasionally, Mc may disseminate into the bloodstream resulting in septicaemia. From the bloodstream, the bacteria might be able to get access to and cross the blood-brain barrier (BBB), initiating meningitis. The progression of systemic Mc disease can be very rapid and disease development primarily affects small children and adolescents, often resulting in serious disease causing neurological sequelae or fatal outcome. The first clinical symptoms of meningococcal disease are headache, stiff neck, rashes and fever, hence it is not so different from the common cold. The rapid progression of this infection makes it extremely difficult to handle. The general condition of the patient might deteriorate after only a few hours once the first symptoms have appeared, thus, a rapid diagnosis and treatment is of paramount importance.

Mc cells are spread from person-to-person by aerosol formation from the airways. Several studies have focused on identifying factors involved in predisposition of meningococcal carriage and disease (Blackwell *et al.*, 1990; Blackwell *et al.*, 1992; Olcen *et al.*, 1981; Stuart *et al.*, 1989; Young *et al.*, 1972). Age, intimate personal contact, crowding and smoking are factors that affect the Mc carriage rate. In addition, damage to the nasopharyngeal mucosal membranes due to co-infections, (passive) smoking, low humidity, dry mucosal membranes and implications due to dust predisposes individuals to carriage and Mc disease (Stephens *et al.*, 2007). Furthermore, the onset of systemic Mc disease particularly occurs in individuals lacking bactericidal antibodies or an efficient complement response (Goldschneider *et al.*, 1969a; Schneider *et al.*, 2007; Stephens *et al.*, 2007). The pathogenetic process in Mc disease is still not well understood, although several outbreaks occur each year (Stephens *et al.*, 2007). The ability to adhere to mucosal epithelial cells in the oro-nasopharynx of humans is a prerequisite for colonization. In addition, studies have shown that *N. meningitidis* adheres to the endothelium of both the meninges and the choroid plexus (Pron *et al.*, 1997). Moreover, the mechanism of meningococcal invasion and passage of the mucosa and BBB is only partially known (Nassif, 2000).

Although closely related, *N. gonorrhoeae* (the gonococcus, Gc) colonizes a different anatomical niche and causes gonorrhea, a type of sexually transmitted disease, which is distinct from the type of disease caused by Mc strains. Gc mainly infects the urogenital epithelium, but other sites of colonization are the rectum, oropharynx and conjunctiva. In addition, some Gc strains may cause systemic infections, however, this occurs less frequently than with Mc strains. The main symptoms of Gc infection are itching, a urethral pus-like discharge and a burning sensation during urination. A Gc infection is not life-threatening, however, an ascending Gc infection in infected women may result in pelvic inflammatory disease (PID) and infertility. Both

men and women may be infected by Gc without having symptoms. Among infected persons, the asymptomatic carriage rate is 50-80% in women, while it is much lower in men (Tao & Irwin, 2006).

Neisseria lactamica is the most commonly occurring neisserial commensal (Holten *et al.*, 1978). Compared to Mc and Gc, it is primarily transferred by the fingers of small children and is shown to contribute to protection against Mc infection, due to the production of crossreactive antibodies (Gorringe *et al.*, 2005; Li *et al.*, 2006).

Table 1. Summary of the most common symptoms, virulence factors and diagnostic tests relevant for meningococcal disease.

Septicaemia	**Meningitis**	**Virulence factors**	**Diagnostic tests**
Fever	Fever	Polysaccharide capsule	Gram stain: Gram-negative diplococci in spinal fluid and/or
Headache	Headache	Type IV pili	blood culture
Chills	Nausea	Opacity proteins	Growth on Thayer-Martin Media
Fatigue	Neck stiffness	OMPs (PorA/B)	Spinal fluid diagnostics:
Hypotension	Vomiting	LOS/Endotoxin	- Fluorescence microscopy
Shock	CNS damage	RTX/Exotoxin	- Latex agglutination with
Purpura	Acute confusion	Immune escape:	anticapsular antibodies
Muscle pain	Chills	- IgA_1 protease	- PCR detection
	Light sensitivity	- Antigenic variation	
	Rashes	- Capsule	

1.1.1 Mc virulence factors

Several virulence factors are associated with systemic Mc disease (Table 1). The most important of these are associated with surface structures and the outer membrane. *N. meningitidis* produces a polysaccharide **capsule** shown to mediate protection against phagocytic killing, opsonization and complement-mediated killing (Gotschlich *et al.*, 1969). Interestingly, bacteria isolated from blood or the cerebrospinal fluid (CSF) of patients with sepsis and meningitis are heavily encapsulated,

while bacteria isolated from the nasopharynx of asymptomatic carriers lack the capsule (Nassif, 2000). Due to the importance of its antiphagocytic properties and the ability of *N. meningitidis* to regulate capsule expression and switching, the polysaccharide capsule is considered to be one of the major Mc virulence factors (Edwards *et al.*, 1994; Hammerschmidt *et al.*, 1994). Moreover, Mc strains of serogroups B, C, Y and W-135 produce capsules composed entirely of polysialic acid or sialic acid linked to glucose or galactose, while the capsule of Mc serogroup A is composed of *N*-acetyl mannosamine-1-phosphate (Bhattacharjee *et al.*, 1976; Liu *et al.*, 1971a; Liu *et al.*, 1971b).

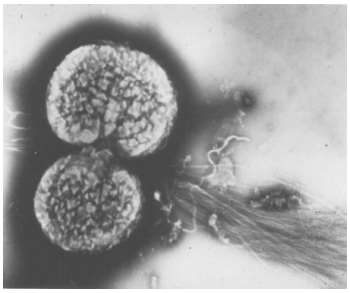

Fig. 1. EM image of piliated *N. meningitidis* H44/76 wildtype strain (Tønjum, 2005).

Mc expresses long, filamentous-like appendages emanating from the bacterial surface, called **type IV pili** (Fig. 1). Type IV pili are particularly important for neisserial survival and virulence (Merz & So, 2000; Meyer *et al.*, 1994). In addition, type IV pilus expression is required for the initial adherence to human epithelial cells (Pujol *et al.*, 1999; Stephens & McGee, 1981; Swanson, 1973), while expression of **opacity proteins** including Opa and Opc mediates a more intimate attachment (Dehio *et al.*, 1998; Nassif *et al.*, 1999). Among the outer membrane proteins (**OMPs**) expressed by Mc strains are the two major porins, PorA and PorB. PorA has a role in negative regulation of the complement response, while PorB facilitates in the close attachment and invasion of host cells (Jarva *et al.*, 2005; Rudel *et al.*, 1996). Studies on the role of PorB have

however shown contradictory results on its role in host cell apoptosis (Massari *et al.*, 2003; Muller *et al.*, 1999).

Like other Gram-negative bacteria, Mc has a cell wall consisting of two membranes and a thin peptidoglycan layer. The inner membrane (IM) consists of proteins embedded in a phospholipid bilayer, while the outer membrane (OM) is asymmetrically made of phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet, in addition to proteins (Bos *et al.*, 2007). *N. meningitidis* expresses lipo-oligosaccharide (**LOS)**, an endotoxin structurally different from the LPS of enteric endotoxins (Kahler & Stephens, 1998). Characteristics of LOS are its oligosaccharide structure and the absence of repeating O-antigen subunits (Griffiss *et al.*, 1988). The severity of inflammation and disease closely correlates with levels of LOS, emphasizing the importance of LOS in neisserial virulence (Stephens *et al.*, 2007). LOS concentrations in plasma and CSF can vary from only a few pg/ml up to 1µg/ml, due to invading bacteria and shedding of outer membrane vesicles (OMVs). Only a few pg LOS is enough to induce inflammatory responses that greatly affect circulation and other vital body functions (Brandtzaeg *et al.*, 2001; Post *et al.*, 2005; Stephens *et al.*, 2007). Furthermore, the structure of LOS has been shown to mediate activation of human macrophages, as well as other host cells, through stimulation of the Toll-like receptor 4 (TLR4). TLR4 induces the subsequent release of several cytokine/chemokine responses, such as TNF-α (Waage *et al.*, 1989), leading to acute inflammation, circulatory collapse and multiple organ failure (Stephens *et al.*, 2007; Zughaier *et al.*, 2004). Mc also expresses several variants of LOS and mimics structures on human cells by LOS sialylation, a process suggested being important in Mc immune escape (Jones *et al.*, 1992; Mandrell & Apicella, 1993).

In addition, Mc produces **IgA_1 protease**, which protects the bacteria from the action of IgA antibodies. IgA is produced and secreted on mucosal surfaces, preventing bacterial attachment and colonization. IgA_1 protease is a sequence-specific peptidase, which cleaves IgA in the hinge region, rendering it non-functional (Plaut, 1983). The level of IgA_1 protease activity was shown to be significantly higher in invasive Mc strains compared to strains isolated from asymptomatic carriers (Vitovski *et al.*, 1999). Furthermore, *Haemophilus influenzae* and *Streptococcus pneumoniae*, both causative agents of bacterial meningitis, also produce IgA_1 protease, indicating a role of IgA_1 protease in the virulence of pathogens colonizing the mucosal surfaces of the human nasopharynx (Kilian *et al.*, 1996; Tunkel & Scheld, 1993).

Mc does not express any known classical exotoxins, however, sequence similarities of Mc FrpA and FrpC proteins to **RTX toxins** have been documented (Thompson & Sparling, 1993; Thompson *et al.*, 1993).

1.1.2 Mc epidemiology

Mc strains are traditionally classified based on antigenic variants of the capsular polysaccharide (serogroup), the OMP PorB (serotype), the OMP PorA (serosubtype) and LOS (immunotype) (Frasch *et al.*, 1985; Tzeng & Stephens, 2000). At present, thirteen Mc serogroups have been identified, however, only six of them (A, B, C, Y, W-135 and X) are associated with major Mc infections and life-threatening disease (Stephens *et al.*, 2007). Serogroup B strains are the main cause of most Mc disease outbreaks in industrialized countries as manifested recently on New Zealand from 2001-2006 (Stephens, 2007). In Norway, the incidence of meningococcal infection and disease culminated in an epidemic plateau during the years of 1975-1979 (Bøvre & Gedde-Dahl, 1980). The Northern part of Norway was most affected by the epidemic, and the highest

incidence of disease occurred during the transition of winter to spring. The epidemic persisted for many years, and not until the late 1980s did the incidence of meningococcal disease decline. Interestingly, the epidemic was mainly due to a Mc serogroup B strain, which was resistant to sulfonamide (Caugant *et al.*, 1986; Caugant *et al.*, 1994). In contrast, Mc serogroup A strains account for the large epidemic outbreaks in Africa (Achtman, 1995). The occurrence of meningococcal disease varies with climate. Disease caused by serogroups A and C is more pronounced during the dry season in Africa, while the number of incidences due to serogroups B and C increases during the winter months in industrialized countries (Stephens, 2007). However, during the last decade, outbreaks of Mc serogroups W-135, X and Y have occurred, emphasizing the importance of effective prevention and treatment also against these serogroups. In 2002, an epidemic outbreak of Mc serogroup W-135 occurred in Burkina Faso, while a significant increase in the incidence of meningitis due to Mc serogroup X has been observed in Niger since the mid 1990s (Boisier *et al.*, 2007; Djibo *et al.*, 2003; Parent du Chatelet *et al.*, 2005).

1.1.3 Mc vaccine development

Knowledge on pathogenesis, virulence and pathophysiology has been the building blocks for development of preventive strategies against meningococcal disease. Although chemoprophylaxis is effective in limited outbreaks, vaccination regimes are the most effective tool in areas with large epidemics (Stephens *et al.*, 2007). Based on studies on the immunoprotective properties of the Mc capsule in the 1960s (Artenstein *et al.*, 1970; Gold *et al.*, 1975; Goldschneider *et al.*, 1969a; Goldschneider *et al.*, 1969b; Gotschlich *et al.*, 1969), effective capsular polysaccharide vaccines were developed to decrease outbreaks of Mc disease due to serogroups A, C, Y and W-135 in the 1970s and 1980s (Morley & Pollard, 2001; Stephens, 2007). The capsular polysaccharide of Mc

serogroup B is poorly immunogenic due to sialylation and structural resemblance to human neural tissues, thus a safe and protective polysaccharide vaccine against this serogroup is still warranted (Bilukha & Rosenstein, 2005; Finne *et al.*, 1983; Kahler *et al.*, 1998). Furthermore, the polysaccharide vaccines do not induce long-term immunological memory and are poorly immunogenic in infants of less than two years, limiting their use in prevention and treatment of Mc disease (Bilukha & Rosenstein, 2005; Stephens *et al.*, 2007).

The development of polysaccharide-protein conjugate vaccines has been more effective in limiting Mc disease, since these vaccines also stimulate the T-cell response, resulting in more long-term memory. In addition, these vaccines are safe, immunogenic in young children, provide long-term protection and decrease nasopharyngeal carriage (Bilukha & Rosenstein, 2005; Stephens *et al.*, 2007). Recently, the tetravalent polysaccharide meningococcal conjugate vaccine, targeting Mc serogroups A, C, Y and W-135, was reported to be as effective as the previously produced polysaccharide vaccines and to provide longer protection against Mc disease (Smith, 2008). In addition, a polysaccharide-protein conjugate vaccine (MenC) against Mc serogroup C was produced and tested in the U.K. at the end of the 1990s. This vaccine proved to be effective in reducing the incidence of Mc disease due to serogroup C among young children and adolescents (Borrow *et al.*, 2002). In addition, administration of the vaccine also decreased carriage rate and transmission of the pathogen (herd immunity) (Snape & Pollard, 2005). However, some meningococcal isolates from MenC vaccinated patients were recently shown to be resistant to bactericidal antibodies elicited by the vaccine. Notably, these isolates had acquired an insertion sequence, IS*1301*, in the *cps* locus, resulting in overproduction of the polysaccharide capsule (Uria *et al.*, 2008). Furthermore, the occurrence of Mc serogroup X outbreaks in Niger is of major

concern due to the fact that no vaccine has been developed against this serogroup (Boisier *et al.*, 2007).

Due to the limitations of Mc serogroup B capsular polysaccharides in vaccine development, strategies to develop additional vaccine candidates are ongoing, focusing on OMP, OMV and LOS surface antigens (Finney *et al.*, 2008; Jäkel *et al.*, 2008; Sardinas *et al.*, 2006; Urwin *et al.*, 2004). An OMV vaccine against Mc serogroup B was developed and tested in Norway in the late 1980s (Bjune *et al.*, 1991), contributing to the current knowledge of such vaccines. Recently, other non-capsular Mc vaccine candidates have been proposed, including LctP and ExbB (Sun *et al.*, 2005a), GNA2132 (Plested & Granoff, 2008), NadA (Comanducci *et al.*, 2002) and GNA1870/fHbp (Schneider *et al.*, 2009). Furthermore, vaccine development has benefited on the completion of neisserial genome sequences, which in turn facilitates reverse vaccinology (Giuliani *et al.*, 2006; Rappuoli, 2001).

1.1.4 Assessing Mc pathogenesis

The establishment of additional human organ studies and animal models would improve our knowledge of Mc pathogenicity. Yet, the fact that Mc exclusively colonizes the human nasopharynx, limits the use and relevance of animal models in these studies (Gorringe *et al.*, 2005; Stephens, 1989). Furthermore, the potential severity of Mc infections limits the use of human volunteers, however, studies on human cells, cell lines and organ cultures, in addition to functional genomics studies, have provided information on Mc cytotoxicity, attachment and invasion (Exley *et al.*, 2009; Stephens, 1989; Sun *et al.*, 2000). Animal models are, however, needed in the primary monitoring of protective and immunogenic Mc vaccine candidates (Gorringe *et al.*, 2005). In fact, the use of CD46 and transferrin transgenic mice ("humanized mice") have revealed new insights

into the pathogenicity of *N. meningitidis* and additional transgenic mice models are warranted (Sjölinder & Jönsson, 2007; Zarantonelli *et al.*, 2007).

1.1.5 Neisserial genome sequences

The complete genome sequences of three hypervirulent Mc strains; Z2491, MC58 and FAM18, representing serogroups A, B and C, respectively, have been published (Bentley *et al.*, 2007; Parkhill *et al.*, 2000; Tettelin *et al.*, 2000). Furthermore, additional genome sequences from representatives of the genus *Neisseria* are continuously becoming available. A whole-genome comparison of Mc, Gc and *N. lactamica* strains revealed new insights into the evolution of virulence in *N. meningitidis* (Schoen *et al.*, 2008). Results from this study suggest that Mc, Gc and *N. lactamica* strains originate from an unencapsulated common ancestor, and that Mc strains have acquired the ability to produce their capsule by the uptake of the *cps* locus through horizontal gene transfer (Schoen *et al.*, 2008). For several clinically important pathogens, pathogenicity relies on strain-specific genes concentrated on the genome, forming so-called pathogenicity islands (Hacker *et al.*, 1997; Hacker & Kaper, 2000). Surprisingly, no such Mc-specific pathogenicity island was found in comparative genomic analysis, including the closely related pathogenic and nonpathogenic neisserial strains *N. meningitidis*, *N. gonorrhoeae* and *N. lactamica* (Perrin *et al.*, 2002). Instead, small strain-specific sequences scattered around the Mc genome were identified. Since the development of systemic meningococcal disease and subsequent death of the host do not favor Mc transmission, the authors hypothesize that Mc is essentially a commensal, and that the ability to cause disease originates from the selection of important colonization factors (Perrin *et al.*, 2002). These results corroborate findings on neisserial population genomes generated by multilocus sequence typing (MLST) (Maiden, 2008). Furthermore, although genetic recombination

among neisserial species has been reported, a MLST study suggests that the frequency of recombination between separate species is low (Bennett *et al.*, 2007). The genome sequences reveal that neisserial species have well-developed DNA repair, recombination and replication (3R) systems, although AlkA and alkylation reversal are missing and the mismatch repair pathway is reduced (no MutH) as compared to *Escherichia coli* (Davidsen & Tønjum, 2006). In general, defects in DNA repair genes can generate mutator phenotypes and affect Mc virulence and the development of antibiotic resistance (Carpenter *et al.*, 2007; Richardson *et al.*, 2002).

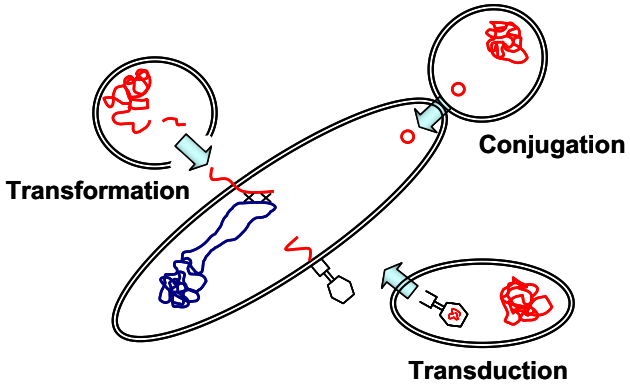

Fig. 2. Schematic overview of horizontal gene transfer processes in bacterial species.

1.2 Horizontal gene transfer

Horizontal gene transfer (HGT) causes much of the abundant diversity seen in many bacterial species. Three types of HGT mediate exchange of DNA between bacterial strains; conjugation, transduction and transformation (Davidson, 1999) (Fig. 2).

Conjugation: the process in which bacterial DNA is moved from one bacterium to another on a self-transmissible conjugative plasmid.

Transduction: the process in which a bacteriophage incorporates bacterial DNA and transfers it to another bacterial cell.

Transformation: the process in which exogenous DNA is bound, taken up and incorporated into the bacterial genome.

Among these HGT processes, transformation is the main source of new DNA introduced into neisserial genomes (Koomey, 1998). Transduction seems to occur rarely in *Neisseria*, although the genome sequences have revealed remnants of prophages (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000). In addition to genome instability caused by transformation and transposable conjugation events, the neisserial genome is hypervariable due to frequent phase and antigenic variation as well as spontaneous intragenomic recombination and mutation events (Davidsen *et al.*, 2007). Bacteria use different kinds of molecular machines in order to take up DNA from its surroundings. In *Neisseria*, the components involved in type IV pilus biogenesis and transformation are related to the type II secretion system, while transformation in *Helicobacter pylori* involves components of the type IV secretion or conjugative DNA transfer systems (Chen & Dubnau, 2003).

1.2.1 Secretory pathways in neisserial species

Bacteria have evolved several pathways for the secretion of proteins and macromolecules. There are currently six secretory systems identified among Gram-negative bacterial species, designated "Type (I – VI) secretion system" (Bingle *et al.*, 2008; Cascales, 2008; Henderson *et al.*, 2004; van Ulsen & Tommassen, 2006). Protein secretion across the two membranes of Gram-negative

bacteria can either occur as a single step or be divided into two separate steps. In the two-step secretion pathway, protein secretion across the inner membrane is conducted either through the Sec (de Keyzer *et al.*, 2003) or the Tat pathway (Palmer *et al.*, 2005; Robinson & Bolhuis, 2004). The **type I** secretion system consists of an inner membrane ATPase (the ABC protein), a membrane fusion protein (MFP) and an outer membrane channel protein. Together, these three proteins mediate direct transport of toxins, proteases and lipases through the cellular membranes. In *N. meningitidis*, the existence of a type I secretion system was manifested by the identification of the RTX proteins FrpA and FrpC (Thompson & Sparling, 1993; Thompson *et al.*, 1993). The **type II** secretion system, also known as the general secretory pathway (GSP) (Pugsley, 1993), is broadly conserved among Gram-negative bacteria and allows the delivery of macromolecules and virulence factors into the surroundings (Johnson *et al.*, 2006; Nunn, 1999). While other Gram-negative bacteria have multiple type II secretion systems, *Neisseria* spp. have only a single system of this kind, which is responsible for type IV pilus biogenesis (Carbonnelle *et al.*, 2006; Chen & Dubnau, 2003; Tønjum & Koomey, 1997). The **type III** secretion system is conserved among different bacterial pathogens and mediates the direct transport of proteins from the bacterial cell into the cytosol of eukaryotic cells (Hueck, 1998). However, *Neisseria* spp. lack a type III secretion system (van Ulsen & Tommassen, 2006). The **type IV** secretion systems are related to conjugation systems (Burns, 1999) and are devoted to the direct secretion of bacterial proteins and/or DNA into target cells (Burns, 2003; Cascales & Christie, 2003). The genomes of most *N. gonorrhoeae* strains, in addition to some *N. meningitidis* strains, have been found to contain a genetic island (GGI) encoding a type IV secretion system (Dillard & Seifert, 2001; Hamilton *et al.*, 2005; Snyder *et al.*, 2005). The Gc type IV secretion system secretes chromosomal DNA and the GC content of GGI indicates that it has been introduced into the Gc genome through horizontal

gene transfer (Dillard & Seifert, 2001; Hamilton *et al.*, 2005). The **type V** secretion system includes the autotransporter and the two-partner secretion (TPS) systems (Henderson *et al.*, 2004; Jacob-Dubuisson *et al.*, 2001). The IgA_1 protease is one of several autotransporters expressed in pathogenic *Neisseria*, and recent studies have emphasized the importance of neisserial TPS systems in virulence (van Ulsen & Tommassen, 2006; van Ulsen *et al.*, 2008). The recently identified **type VI** secretion system was first characterized in *Vibrio cholerae* and *Pseudomonas aeruginosa* (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). Gene clusters related to type VI secretion were originally identified due to the presence of an *icmF* (intracellular multiplication) homolog, engaged in the pathogenesis of *Legionella pneumophila* (Bingle *et al.*, 2008; Zusman *et al.*, 2004). These clusters are usually found within pathogenicity islands or on chromosomes dedicated to bacterial virulence or survival. Proteins secreted by this pathway lack N-terminal signal sequences and are consequently not secreted by the Sec pathway (Bingle *et al.*, 2008; Cascales, 2008). Many bacterial species possess multiple copies of the type VI secretion clusters and whether or not these clusters arise from duplication or gene transfer is not known (Bingle *et al.*, 2008; Cascales, 2008). The presence of orthologs of the type VI secretion system in the genomes of pathogenic *Neisseria* have not been detected (Shrivastava & Mande, 2008). Thus, the pathogenic neisserial species harbor four distinct pathways for the secretion of proteins and macromolecules into their external surroundings; the type I, II, IV and V secretion systems (Table 2).

Table 2. Summary of protein secretion systems identified in Gram-negative bacteria.

Protein secretion system	General description	Present in *Neisseria*
I	Direct secretion of toxins, proteins and lipases	+ RTX toxins
II	Secretion of virulence factors by the use of pilus-like structures (pseudopili)	+ Type IV pili
III	Direct transport of proteins between the bacterial cytoplasm and the target cell	-
IV	Direct transport of proteins and/or DNA into the target cell	+ GGI
V	Secretion of autotransporters and TPS-associated proteins	+ IgA_1 protease
VI	Protein secretion independent on signal sequence or the Sec pathway	-/?

1.2.2 Type IV pilus biogenesis

Neisserial type IV pili are long and thin filamentous-like appendages consisting of thousands of pilin subunits in addition to pilus-associated proteins (Dalrymple & Mattick, 1987). Studies have shown great structural similarities among the family of type IV pilins and the structure of the neisserial major pilin protein PilE has been resolved, contributing to a more comprehensive understanding of the structure and function of this important virulence factor (Parge *et al.*, 1995; Strom & Lory, 1993). Notably, the neisserial type IV pilus biogenesis machinery is a highly conserved biogenesis pathway, that resembles the type II protein secretion system (Nunn, 1999). While a number of different fimbrial types can occur on the surface of other bacteria, *Neisseria* express only type IV pili. Type IV pilus biogenesis is conducted by a complex set of proteins forming the pilus biogenesis apparatus (Tønjum & Koomey, 1997). In *N. meningitidis*, this process is dependent on the expression of 15 proteins (Carbonnelle *et al.*, 2005), some important in pilus assembly and others in pilus stability (Carbonnelle *et al.*, 2006). Studies on type IV pilus biogenesis suggest the existence of a multi-step process including pilus assembly in the periplasm,

stabilization and maturation, followed by surface exposure by pilus extrusion through the secretin PilQ (Carbonnelle *et al.*, 2006; Wolfgang *et al.*, 2000).

The expression of type IV pili is associated with several phenotypes, such as competence for transformation (Frøholm *et al.*, 1973; Swanson *et al.*, 1971), adherence (Swanson *et al.*, 1971; Swanson, 1973), twitching motility (Henrichsen *et al.*, 1972; Mattick, 2002), biofilm formation (O'Toole & Kolter, 1998), bacteriophage infection (Bradley, 1974), and virulence (Bieber *et al.*, 1998; Comolli *et al.*, 1999; Merz *et al.*, 1999; Pujol *et al.*, 1999). Type IV pili are vital for adherence and are believed to play an important role in the initial stage of infection by mediating adherence between the associated PilC proteins, PilC1 and PilC2, and human epithelial and endothelial cells (Jönsson *et al.*, 1994; Nassif *et al.*, 1994; Rudel *et al.*, 1995). Furthermore, twitching motility is the bacterial movement mediating intimate adherence caused by type IV pilus retraction (Merz & So, 2000), powered by the inner membrane ATPase PilT (Wolfgang *et al.*, 1998).

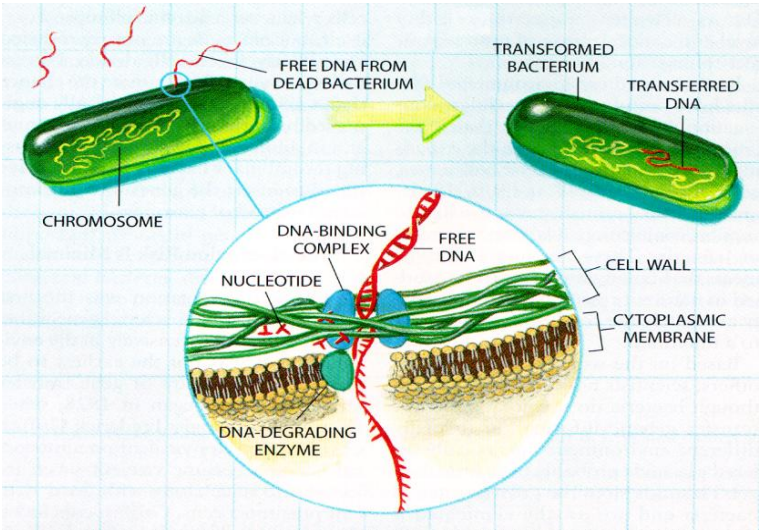

Fig. 3. The transformation process in bacteria (Miller, 1998).

1.3 Competence for transformation

In contrast to other Gram-negative bacteria, *N. meningitidis* is naturally competent for transformation throughout its life cycle (Jyssum & Lie, 1965) (Fig. 3). The neisserial transformation process is dependent on type IV pilus expression (Swanson *et al.*, 1971), the presence of the neisserial DNA uptake sequence (DUS) in the incoming DNA (Ambur *et al.*, 2007; Goodman & Scocca, 1988; Mathis & Scocca, 1984) and RecA-dependent homologous recombination (Koomey & Falkow, 1987). The uptake of DNA through natural transformation can be dissected into several steps, encompassing the binding of DNA, the uptake of DNA into a DNase-resistant state, translocation over the membranes and peptidoglycan layer and genome incorporation (Aas *et al.*, 2002b). Although the process of DNA uptake has been studied to some extent in *N. gonorrhoeae*, very little is known about the uptake of exogenous DNA into neisserial cells (Davidsen & Tønjum, 2006; Koomey, 1998).

1.3.1 The DNA uptake sequence (DUS)

N. meningitidis preferentially takes up its own DNA or that of closely related species. This is conducted through the recognition of a 10-bp DNA uptake sequence termed DUS (5′ GCCGTCTGAA 3′) (Goodman & Scocca, 1988). However, recent studies have established the preference for a 12-bp DUS within the neisserial species, where the 10-bp DUS is extended by a 5'-AT (Ambur *et al.*, 2007). The completion of the genome sequence projects on Mc strains MC58 and Z2491, revealed the existence of approximately 2000 copies of DUS evenly distributed throughout the neisserial genome (Parkhill *et al.*, 2000; Smith *et al.*, 1999; Tettelin *et al.*, 2000). Furthermore, DUS located inside ORFs are biased towards 3R genes, indicating a role of transforming DNA in genome maintenance (Davidsen *et al.*, 2004; Treangen *et al.*, 2008).

Prior to the discovery of DUS, the naturally competent bacterium *H. influenzae* was shown to harbor a DNA uptake sequence termed USS (Mathis & Scocca, 1982). Notably, the USS sequence has a different composition (5′ AAGTGCGGT 3′) than the neisserial DUS (Mathis & Scocca, 1982; Smith *et al.*, 1999). Studies have revealed the occurrence of HGT events between *Haemophilus* and neisserial strains, indicating the ability of DNA transfer between bacteria harboring the same ecological niche (Kroll *et al.*, 1998). In addition, a high frequency of DNA exchange between Mc and commensal neisserial strains has been reported (Linz *et al.*, 2000).

1.4 DNA binding proteins

Interactions between proteins and DNA are crucial for several cellular processes, such as 3R gene activities, transcription and HGT including transformation. DNA binding proteins harbor different types of folded patterns mediating their DNA binding abilities. These folded patterns, also termed DNA binding motifs, comprise a recognition region and a stabilization region. Furthermore, protein-DNA interactions are either non-specific, interactions between the positively charged protein side-chains and the negatively charged DNA sugar/phosphate backbone, or specific, interactions between protein side-chains and nucleotide bases. The most well-known recognized DNA binding motifs are the following:

Helix-turn-helix (HTH): HTH is the most studied DNA binding motif and regulatory proteins harboring this motif are found among both prokaryotes and eukaryotes (Harrison & Aggarwal, 1990). The difference between the prokaryotic and eukaryotic HTH proteins is that the latter are able to interact as both monomers and/or heterodimers, while the prokaryotic HTH proteins bind DNA as dimers. The HTH motif is traditionally defined as a 20 amino acid segment

of two perpendicular α-helices separated by a β-turn, where one of the helices (the recognition helix) enters the major groove of the DNA (Luscombe *et al.*, 2000).

Zinc finger: Zinc fingers are common among eukaryotic transcriptional regulators and the widespread use of this motif is believed to be due to the structural stability mediated by zinc ions. One characteristic of zinc fingers is the tetrahedral coordination of zinc ions by conserved cysteine and histidine residues (Luscombe *et al.*, 2000). Notably, the use of zinc finger motifs is not restricted to DNA binding, since these motifs are also found in domains mediating interactions between proteins (Mackay & Crossley, 1998). Furthermore, proteins often contain multiple zinc fingers and the number of fingers is thought to contribute to DNA binding specificity (Luscombe *et al.*, 2000).

Leucine zipper: The leucine zipper is composed of two α-helices that interact through leucines and nonpolar residues, forming a dimer. The C-terminal of each helix mediates dimerization, while the N-terminal mediates DNA binding. Thus, the α-helices of a leucine zipper dimer bind to the major grove of DNA in opposite directions (Luscombe *et al.*, 2000). The leucine zipper family consists entirely of the yeast GCN_4 prototype proteins and their homologs, which interact with promoter regions of genes involved in amino acid biosynthesis (Ellenberger *et al.*, 1992).

Helix-loop-helix: The helix-loop-helix motif mediates dimer formation and in almost all cases a heterodimer of two such proteins is required for DNA binding activity (Harrison, 1991).

Generally, the DNA binding properties of a DNA binding protein are influenced by the chemical and physical properties of the DNA-protein interface. Characterization of various DNA binding motifs demonstrates that the majority of DNA binding motifs bind DNA in the major groove, while binding to the minor groove involves distortion of the DNA structure (Jones *et al.*,

1999). Furthermore, DNA binding motifs are, in general, amongst the most positive electrostatic patches on the surface of proteins (Jones *et al.*, 2003). Interestingly, while a DNA binding motif is located on a single polypeptide chain, many DNA binding proteins mediate their function through dimerization or multimerization. In particularly, dimerization contributes to stability and functional efficacy (Marianayagam *et al.*, 2004).

1.5 Pilus biogenesis components and other non pilus-related neisserial competence factors

The neisserial transformation process is dependent on the expression and activity of several pilus-related components, including pilus biogenesis components (Tønjum & Koomey, 1997) and the minor pilin **ComP** (Wolfgang *et al.*, 1999). Among the pilus biogenesis components required for transformation are the outer membrane protein **PilQ** (Collins *et al.*, 2005; Frye *et al.*, 2006) and the inner membrane proteins **PilG**, **PilP** and **PilT** (Balasingham *et al.*, 2007; Tønjum *et al.*, 1995; Wolfgang *et al.*, 1998). PilQ belongs to the large family of secretins, also known as the GspD family (Pugsley, 1993). PilQ monomers multimerize into a dodecamer structure forming an outer membrane pore, through which pili are extruded and retracted (Collins *et al.*, 2005). The topology of Mc PilQ has been proposed, revealing new insights into the structure and interactions between Mc membranes (Frye *et al.*, 2006). The PilQ multimer is stabilized by the lipoproteins PilW and PilP, for which the 3D structures have been resolved (Carbonnelle *et al.*, 2005; Golovanov *et al.*, 2006; Trindade *et al.*, 2008). Moreover, PilQ is so far the only pilus biogenesis component shown to possess DNA binding activity (Assalkhou *et al.*, 2007). PilG belongs to the GspF family of proteins, which comprises inner membrane proteins conserved among Gram-negative bacteria, involved in type II secretion and type IV pilus biogenesis (Collins *et al.*, 2007; Tønjum *et al.*,

1995). The exact function of PilG has not been elucidated, however, a low resolution 3D structure of PilG has been shown (Collins *et al.*, 2007). PilP was first thought to function as a pilot protein stabilizing PilQ (Drake *et al.*, 1997). Recent studies, however, suggest that PilP is necessary for pilus biogenesis and might have additional functions conducted through its interactions with PilQ (Balasingham *et al.*, 2007; Carbonnelle *et al.*, 2006). PilT is required for type IV pilus retraction in Gram-negative bacteria. PilT mutants have been shown to hyperagglutinate and lack the ability of twitching motility (Wolfgang *et al.*, 1998). Interestingly, Δ*pilT* mutants are not deficient of pilus expression, but are unable to take up exogenous DNA through transformation, implying that pilus retraction is required for DNA uptake (Wolfgang *et al.*, 1998). PilT has been shown to form hexameric ring structures and possess ATPase activity (Forest *et al.*, 2004). In addition, PilT shares both structural and functional similarities with NTPases from type II and type IV secretion systems (Planet *et al.*, 2001). The PilT hexamer, as well as the ATPases PilF and PilU, are proposed to be part of a membrane assembled complex (Craig & Li, 2008; Hwang *et al.*, 2003). Moreover, the PilC proteins have been shown to regulate the function of PilT, thus regulating the balance between pilus retraction and extrusion (Morand *et al.*, 2004). ComP has been shown to play a role in transformation, since Δ*comP* mutants show severe defects in DNA uptake (Wolfgang *et al.*, 1999). Interestingly, no alteration in type IV pilus biogenesis or expression of type IV pilus-associated properties such as autoagglutination, twitching motility and cell adherence was observed in these mutants. Furthermore, ComP is expressed in very low amounts *in vivo* and is inhibited by PilV (Winther-Larsen *et al.*, 2001; Aas *et al.*, 2002a).

Among the non pilus-related components, suggested to be involved in neisserial transformation, are the competence factors **ComA** (Facius & Meyer, 1993), **ComE** (Chen & Gotschlich, 2001), **ComL** (Fussenegger *et al.*, 1996a) and **Tpc** (Fussenegger *et al.*, 1997). ComA

is predicted to be an inner membrane protein involved in transport of DNA into the cytoplasm (Facius & Meyer, 1993). Further studies of the ComA ortholog in *Bacillus subtilis*, ComEC, support the notion that it functions as a channel for DNA transport through the cytoplasmic membrane (Draskovic & Dubnau, 2005). ComE has been proposed to be localized in the periplasm and was suggested to bind DNA after DUS-specific DNA uptake has occurred (Chen & Gotschlich, 2001). DNA binding activity of the ComE ortholog in *B. subtilis*, ComEA, has been mapped to its C-terminal, which has a helix-turn-helix motif and therefore shows similarity to proteins known to interact with DNA (Provvedi & Dubnau, 1999). The gonococcal *comL* gene exists as a single copy gene localized downstream of *comA*, sharing transcriptional terminators, and encodes a periplasmic lipoprotein. Reports have suggested that ComL contributes to cleavage of the peptidoglycan layer, providing access for incoming DNA during transformation. A significant decrease in the transformation rate of gonococcal Δ*comL* mutants has been documented, however, the main function of ComL is difficult to assess since most mutants of this essential component are lethal (Fussenegger *et al.*, 1996a). The ComL ortholog in *E. coli*, YfiO, is an OM-anchored lipoprotein and a member of the β-barrel assembly machinery (the BAM complex) (Wu *et al.*, 2005). A recent review refers to ComL as the ortholog of BamD and suggests that neisserial ComL is associated to the OM and involved in OMP biogenesis (Knowles *et al.*, 2009). However, the function of ComL is still unknown and the precise localization of neisserial ComL has not been determined. Orthologs of the *comL* gene are found in *N. meningitidis* as well as in several commensal neisserial species (Fussenegger *et al.*, 1996a). Tpc is involved in peptidoglycan remodeling and Δ*tpc* knock-out mutants are non-competent. Tpc might therefore mediate the passage of DNA through the peptidoglycan layer (Fussenegger *et al.*, 1996b).

1.6 Major DNA repair components related to transformation

The necessity to genetically adapt and evolve in a constantly changing environment requires the existence of multiple DNA repair pathways ensuring the maintenance of bacterial genomes (Davidsen & Tønjum, 2006). Transformation is inherently linked to DNA repair and particularly recombination, which both are processes of genome maintenance (Tønjum *et al.*, 2004). In particular, genome integration of the incoming DNA is dependent on RecA-dependent homologous recombination.

The single-stranded DNA binding (SSB) proteins are conserved and indispensable for cell survival among both prokaryotes and eukaryotes (Fanning *et al.*, 2006; Shereda *et al.*, 2008; Zou *et al.*, 2006). The SSB protein of *E. coli* is involved in processing the frequently occurring single-stranded DNA intermediates in DNA replication, repair and recombination (Shereda *et al.*, 2008). Furthermore, the single-stranded DNA binding activity was shown to reside at the C-terminal part of SSB and the structure of the DNA binding domain has been resolved (Savvides *et al.*, 2004). In addition, SSB orthologs of *B. subtilis* and *H. influenzae* have been linked to transformation (Lindner *et al.*, 2004; Ogura *et al.*, 2002; Redfield *et al.*, 2005). Thus, based on the broad conservation of the structure and function of SSB proteins, the neisserial SSB protein might also have a role in transformation, in addition to recombination and DNA repair.

Generally, there is a need to identify all DNA processing components involved in the neisserial transformation process.

2. Aim of the study

Horizontal gene transfer, recombination and mutational events mediate the abundant genetic diversity observed among neisserial species. Transformation is the main source of incorporation of exogenous DNA into the neisserial chromosome, and although the transformation pathway has been described to some extent, little is known about how the transforming DNA is taken up into the neisserial cell.

The main aim of this study was to identify and characterize DNA binding components involved in neisserial transformation. Based on the already established fact that the PilQ complex pore binds DNA and is a major player in pilus extrusion/retraction and the uptake of exogenous DNA through the outer membrane, the main focus of this work was on inner membrane proteins. The study was conducted to achieve the following subgoals:

- To identify and characterize neisserial DNA binding components co-purifying with the inner membrane
- To optimize methods for searching for DNA binding components within neisserial cellular fractions
- To characterize PilG and ComL and their DNA binding sites
- To characterize the interactions of PilG with other pilus biogenesis components

3. Summary of results

N. meningitidis is naturally competent for transformation throughout its life cycle. In order to elucidate how DNA is transported across the neisserial membranes, we searched for DNA binding proteins within the inner membrane (IM) and soluble cell fraction in a panel of neisserial strains. The cellular fractions were subjected to a solid phase overlay assay with DNA substrates. Subsequently, proteins with DNA binding activity were identified by mass spectrometry (MS) analysis (**Papers I and II**). The resolution of the detection of DNA binding proteins was improved by employing 2D gel electrophoresis, in combination with the solid phase overlay assay and MS analysis, on the neisserial cell fractions (**Paper II**). In the IM, multiple components bound DNA, including the competence lipoprotein ComL, the pilus biogenesis component PilG, the cell division ATP-binding protein FtsE and four hypothetical proteins. In the soluble fraction, a single DNA binding component was predominant and identified as the meningococcal ortholog of the single-stranded DNA binding protein, SSB. Other DNA binding components detected, although with less reproducibility, were PilM, PilN, PilO, PilC1 and RecA. The outer membrane proteins PilQ, H.8 and Omp85 were also found to bind DNA, representing membrane fraction contaminants or proteins interacting with IM proteins. The DNA binding activity of either of these components was not enhanced by the presence of the neisserial DNA uptake sequence, (DUS), indicating that none of these was the DUS-specific receptor. Null mutants, corresponding to each of the proteins identified, were constructed to assess their pilus-related phenotypes. Among the IM proteins identified, only the Δ*pilG* null mutant, defective in pilus biogenesis, was non-competent and non-piliated. This is the first report demonstrating an interaction between PilG and DNA, suggesting that PilG might be directly involved as a player in neisserial transformation, and not only indirectly, through pilus biogenesis. The Δ*comL* and Δ*ssb* null mutants were non-viable. The

DNA binding activity of ComL was analyzed in more detail, verified by electromobility shift analysis (EMSA) of the purified recombinant ComL protein and shown to be Mg^{2+}-dependent (**Paper II**).

A number of components are required for neisserial type IV pilus biogenesis, including the IM protein PilG. PilG mutants are devoid of pili and are non-competent for transformation. Here we found that natively purified and recombinant PilG, in addition to its role in pilus biogenesis, directly binds DNA (**Papers I and II**). In order to characterize PilG further, PilG full-length and partial recombinant proteins were constructed and purified to homogeneity (**Paper III**). By using a solid phase overlay approach with DNA substrates, the DNA binding activity was localized to the N-terminal part of PilG. The DNA binding activity was verified by EMSA analysis and mapped by endoproteinase cleavage of $PilG_{1\text{-}80}$, in combination with MS analysis. PilG DNA binding activity was mapped to residues 1 to 30 and 60 to 80, which we suggest act together to form a DNA binding site. These findings were supported by predictive 3D bioinformatics analyses, which suggest that these residues are closely located in the folded conformation. PilG was also assessed with regard to its multimer formation and direct interaction with other pilus biogenesis components. PilG exhibited a tendency for dimer and tetramer formation. In addition, N-terminal PilG was found to interact with N-terminal PilQ and full-length ComL. Thus, the N-terminal PilG domain was shown to mediate multiple interactions and functions. Even though orthologs of PilG are found in Gram-positive and Gram-negative bacterial species, the N-terminal part of PilG exerting interaction with PilQ is only partly conserved, while the very N-terminal domain contributing to DNA binding is not at all conserved in PilG orthologs, suggesting that the PilG DNA binding is a property specific only to neisserial and closely related species. Taken together, these findings suggest a potential role of both ComL and PilG in neisserial transformation.

4. Discussion

DNA metabolism is comprised of multiple cellular processes, where DNA binding proteins play important roles. Uptake of DNA is of crucial importance for bacterial fitness and survival, yet little is known about the structure and function of DNA uptake machineries. Horizontal gene transfer, in the form of transformation, is the main source of DNA taken up in the naturally competent bacterium *Neisseria meningitidis*. In order to elucidate the process of neisserial DNA uptake, we searched for DNA binding proteins within neisserial cellular fractions. *N. meningitidis* hosts abundant DNA repair, recombination and replication (3R) processes. Taken together, all of these pathways require the presence of a multitude of DNA repair proteins. When conducting an open search for DNA binding proteins, components involved in all of these processes can be detected. In this context, we wanted to identify components involved in transformation. The DNA binding proteins that were reproducibly identified were selected for further characterization of their predicted structure and their functional relationships to type IV pili, other pilus biogenesis components and competence for transformation. These studies revealed new insights into the neisserial transformation pathway and led us to propose an improved model of the neisserial DNA uptake machinery.

4.1 On the relative contribution of neisserial HGT processes

Transformation provides the uptake and replacement of DNA by homologous recombination, while conjugation mediates transfer of extrachromosomal DNA. Furthermore, by transduction foreign (or new) DNA is inserted into host chromosomes. *N. meningitidis* is naturally competent for transformation throughout its life cycle. In addition to recombination, random mutational

events and phase variation, the constant exchange of chromosomal DNA is likely to contribute to the formation of the abundant antigenic diversity observed among neisserial species. The constant genetic variability may contribute to adaptation and improved bacterial fitness and persistence of neisserial infections. In addition to spreading alleles of genes encoding surface markers and evasion of the immune response, transformation facilitates the spread of antibiotic resistance (Spratt, 1994). Conjugation is not widespread in *N. meningitidis*, and only a few *N. meningitidis* strains possess the genes encoding the type IV secretion process that facilitates conjugation in *N. gonorrhoeae*. Only a few bacteriophages have been identified in *N. meningitidis*, displaying different distributions of bacteriophages between hypervirulent and non-invasive isolates (Hotopp *et al.*, 2006). One virulence-associated phage is suggested to be secreted through the secretin PilQ (Bille *et al.*, 2005; Bille *et al.*, 2008). Although transformation mediates antibacterial drug resistance, such as penicillin resistance, due to recombination of new DNA taken up (Spratt, 1994), conjugation and transduction events, in bacteria in general, have been shown to contribute more efficiently in this respect, more frequently generating drug resistance (Lawrence & Hoeprich, 1975). Interestingly, DNA transfer mediated through membrane blebs has been previously documented in *N. gonorrhoeae*, revealing a potentially new genetic exchange mechanism for the spread of antibiotic resistance (Dorward *et al.*, 1989).

4.2 DNA binding properties of pili and the secretin PilQ

The uptake of exogenous DNA into the meningococcal cell during transformation is a multi-step process. Some information exists on the uptake of DNA in *Neisseria*, however, little is known about meningococcal proteins involved in DNA binding and uptake (Davidsen & Tønjum, 2006; Koomey, 1998). The outer-most part of the neisserial DNA uptake machinery is the cell surface,

from which the multi-functional type IV pilus filaments emanate. Due to the requirement of type IV pilus expression for efficient neisserial transformation, we have proposed the hypothesis that neisserial transformation is coupled to pilus retraction and that transforming DNA is non-specifically attached to retracting pili or introduced into the cell in the wake of the retracting pilus (Davidsen & Tønjum, 2006; Tønjum *et al.*, 2004). A similar model has been suggested for *Pseudomonas stutzeri* (Graupner *et al.*, 2001).

Despite the production of type IV pili in *P. aeruginosa*, no evidence for natural transformation has been found in this species, in contrast to *P. stutzeri* which is naturally competent (Carlson *et al.*, 1983). One burning question is whether the pili themselves directly bind DNA. Intriguingly, *P. aeruginosa* type IV pilus-mediated DNA binding was observed in a solution-based assay, a phenotype which was dependent on the expression of intact pili (van Schaik *et al.*, 2005). However, based on previous studies in our laboratory, interactions between pili purified from *N. meningitidis* and *P. aeruginosa* and DNA were reported to be weak, using the same method as van Schaik and co-workers (Assalkhou *et al.*, 2007). Furthermore, purified pili from *N. meningitidis* and *P. aeruginosa* did not exhibit DNA binding activity, when tested in an EMSA analysis. Previously, Mathis and Scocca reported that purified neisserial pili lack DNA binding activity in a solid phase overlay approach (Mathis & Scocca, 1984). The contradictory results on pilus-mediated DNA binding in *N. meningitidis* and *P. aeruginosa* may partly be explained by the methods used, the solution-based assay being more reliable for assessing the long polymerized pilus fibres, than solid phase overlay and EMSA analysis. Interestingly, positively charged patches on the surface of pili from *P. aeruginosa* have been proposed to be responsible for the pilus-mediated DNA binding reported (van Schaik *et al.*, 2005) and similar patches have been predicted on the assembled pili of neisserial species (Parge *et al.*, 1995). Whether the pilus-

mediated DNA binding observed in *P. aeruginosa* indicates that this bacterial species is competent or utilize pilus-DNA interactions in other pilus-associated processes, such as biofilm formation, is not known (van Schaik *et al.*, 2005).

In the outer membrane, the PilQ complex has been shown to be required for transformation through pilus biogenesis (Tønjum *et al.*, 1998). PilQ also binds DNA directly, as previously demonstrated by using three independent methods (Assalkhou *et al.*, 2007). Furthermore, a three-dimensional (3D) reconstruction of the PilQ-PilP interacting complex reveals the possible existence of a PilQ-PilP outer membrane channel through which DNA can enter the neisserial cell (Balasingham *et al.*, 2007). Interestingly, PilP was shown to be localized in the neisserial inner membrane, suggesting that the PilQ complex spans the periplasmic space.

4.3 The DNA binding proteins identified in this study

Based on this study, multiple DNA binding proteins appear to be localized to the neisserial inner membrane. Proteins involved in transformation, cell division and proteins with unknown function were shown to exhibit DNA binding activity (**Papers I and II**) (Table 3). However, among the inner membrane components detected, only the pilus biogenesis component PilG was shown to have a role in transformation, while ComL could not be tested due to its essential character (**Paper I**). Interestingly, two of the hypothetical proteins identified, NMB0478 and NMB0086, harbor DUS within their gene sequences. Since the presence of DUS are biased towards genome maintenance genes (Davidsen *et al.*, 2004), the presence of DUS in these genes might indicate a functional role for these components in DNA metabolism. Furthermore, the *comL* gene has two DUS sequences located in its transcriptional terminator (Jose *et al.*, 2003).

Table 3. Phenotypic characteristics of null mutants corresponding to DNA binding proteins identified by solid phase overlay assay and mass spectrometry (MS) analysis.

Protein identified	Putative function	Colony morphology	Extracellular pilus expression	Competence for transformation
Inner membrane fraction				
NMB0333[1] **PilG** DUS -	Type IV pilus biogenesis	agg- [2]	Absent	Non-competent (Tønjum *et al.*, 1995)
NMB1808 PilM DUS -	Type IV pilus biogenesis	nd [3]	nd	nd
NMB1809 PilN DUS -	Type IV pilus biogenesis	nd	nd	nd
NMB1810 PilO DUS -	Type IV pilus biogenesis	agg-	Absent	Non-competent (Drake *et al.*, 1997)
NMB0007 **FtsE** DUS -	Cell division	agg+	Wt [4]	Competent
NMB0703 **ComL** DUS -	Competence	Not viable	Not viable	Not viable
NMB0478 **Hypothetical protein** DUS+	Unknown	agg+	Wt	Competent
NMB0086 **Hypothetical protein** DUS +	Unknown	agg+	Wt	Competent
NMB1796 **Hypothetical protein** DUS -	Predicted flavoprotein	agg+	Wt	Competent
NMB1963 **Hypothetical protein** DUS -	Periplasmic transport protein	agg+	Wt	Competent
Soluble fraction				
NMB1460 **SSB** DUS -	Single-stranded DNA binding	Not viable	Not viable	Not viable
NMB1445 RecA DUS -	Recombination, DNA repair	agg+	Wt	Non-competent (Tønjum *et al.*, 1995)
Outer membrane fraction				
NMB1812 PilQ DUS -	Secretin, Type IV pilus biogenesis	agg-	Absent	Non-competent (Tønjum *et al.*, 1998)
NMB1847 PilC1 DUS -	Type IV pilus adhesin	agg+	Wt	Competent (Morand *et al.*, 2001)
NMB1533 H.8 DUS -	Iron-sulfur binding	agg+ Smaller than wt	Wt	Competent
NMB0182 Omp85 DUS -	OMP assembly	Not viable	Not viable	Not viable

[1] DNA binding components reproducibly detected in the IM and soluble fractions are indicated in bold.
[2] Colony morphology is described as agglutinating (agg+) or non-agglutinating (agg-).
[3] nd, not done.
[4] Wt, wildtype levels.

Whether the DNA binding activity of the cell division ATP-binding protein FtsE is biologically significant or not requires further studies. Interestingly, a role of FtsE in the balance between DNA condensation and segregation during cell division has been proposed (Bernatchez *et al.*, 2000).

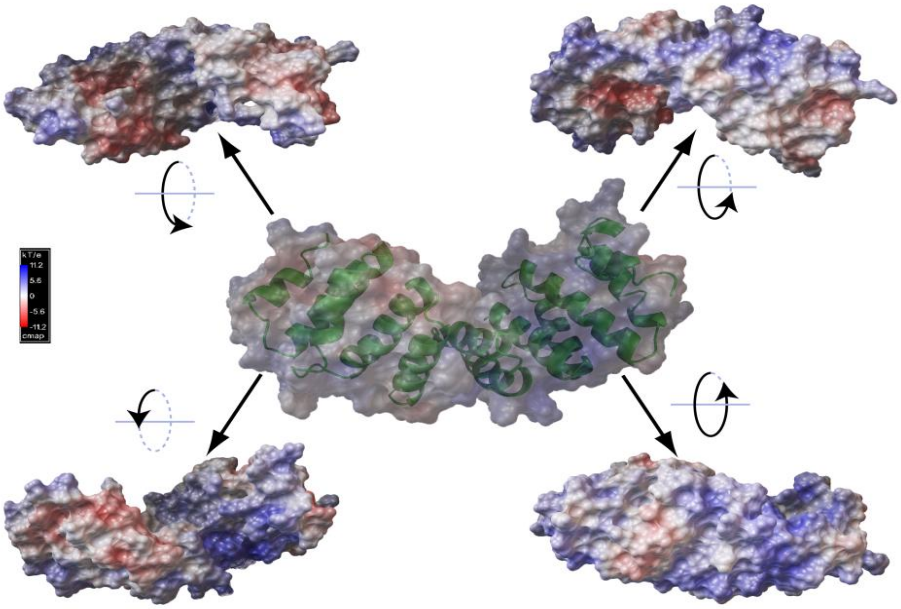

Fig. 4. Predicted 3D structure of Mc ComL. The *N. meningitidis* (Mc) ComL protein is shown in the centre, with the concurrent surface representation overlaid. The charge distribution is shown on the surface, where blue represents positive and red represents negative charge. The large areas of positive charge may play a role in DNA binding.

During transformation, in addition to passing through the membranes, the DNA has to traverse the peptidoglycan layer on its way into the cytoplasm. Interestingly, ComL was found to exhibit DNA binding activity in two independent studies (**Papers I and II**). ComL was shown to interact with both single-stranded and double-stranded DNA, and the observed DNA binding was not enhanced by the presence of DUS. Furthermore, the DNA binding activity of ComL was shown to be Mg^{2+}-dependent, indicating that Mg^{2+} facilitates the direct binding of DNA (**Paper II**). The predicted 3D structure of ComL exhibits multiple sites that potentially could contribute to DNA binding (Fig. 4). Considering the requirement of ComL for efficient gonococcal transformation, ComL, in addition to Tpc, might be involved in the transport of DNA through the periplasm (Facius *et al.*, 1996; Fussenegger *et al.*, 1997). Interestingly, despite its lack of sequence

homology, based on our secondary structure predictions, ComL showed structural homology to *P. aeruginosa* PilF (Kim *et al.*, 2006), which in turn is an ortholog of the neisserial protein, PilW (Trindade *et al.*, 2008).

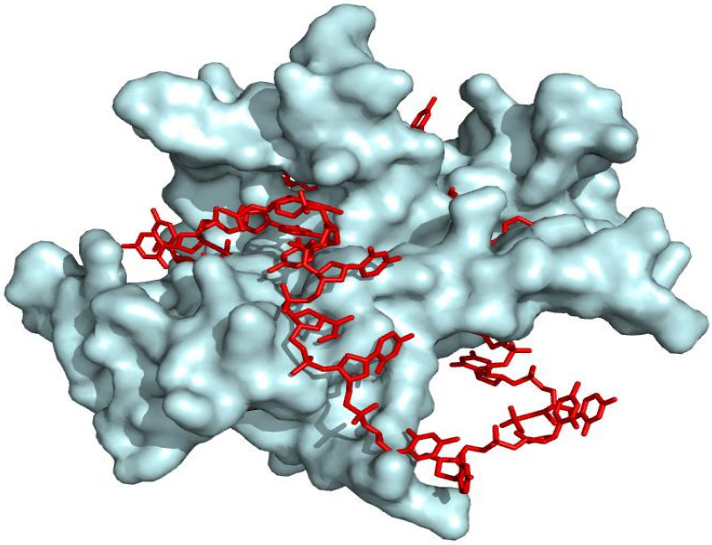

Fig. 5. Predicted 3D structure of Mc SSB. The *N. meningitidis* SSB protein is shown as a dimer, interacting with single-stranded DNA.

Another DNA binding protein detected was the meningococcal ortholog of the single-stranded DNA binding protein SSB (Fig. 5). Since SSB is involved in recombination, it is also relevant for the efficiency of transformation (Lindner *et al.*, 2004; Ogura *et al.*, 2002; Redfield *et al.*, 2005). In our hands, neisserial SSB bound to single-stranded as well as double-stranded DNA without DUS specificity (**Paper II**). While the propensity of SSB to bind single-stranded DNA is well documented, very little is known about its interactions with double-stranded DNA (Shereda *et al.*, 2008). However, studies indicate that particularly prokaryotic SSB might interact with double-stranded DNA during DNA annealing (Makhov & Griffith, 2006; Makhov *et al.*, 2009; Mapelli *et al.*, 2005). Furthermore, RecA has been recognized as an essential factor in the recombinational

part of the transformation process (Koomey *et al.*, 1987). The requirement of both RecA and SSB for efficient transformation might indicate how the recombination and transformation processes are linked.

In summary, multiple DNA binding proteins in the neisserial inner membrane were identified, including the pilus biogenesis component PilG, the competence protein ComL, the cell division ATP-binding protein FtsE and four hypothetical proteins, in addition to the single-stranded DNA binding protein SSB, which was the predominant protein detected in the soluble fraction. The pilus biogenesis proteins PilN, PilM, PilO, PilC1 and the recombinase RecA protein were also detected, however, these findings were not as reproducible as the components described in more detail. In addition, some contamination of outer membrane proteins was also detected, confirming the previously established DNA binding activity of PilQ, in addition to H.8 and Omp85. Whether the DNA binding activity of these components detected *in vitro* is biologically significant, must also be assessed in other systems, and the DNA binding detected must also be viewed in light of the relative abundance of these proteins. The ultimate goal was to identify and locate the hypothesized DUS-specific receptor (Goodman & Scocca, 1988). This was shown to be a difficult task, but the search for the putative DUS receptor will continue in future studies.

Tang and co-workers searched for components involved in meningococcal transformation by a signature-tagged mutagenesis (STM) approach (Hensel *et al.*, 1995). By searching for lack of competence in a mutant library, null mutants lacking the proteins PilN, RecG, DprA, PtsI and Rho were detected (Sun *et al.*, 2005b). Although the STM approach represents a different strategy than ours and is a genetic approach, assessing the lack of gene function, some common factors were detected by the STM and our solid phase overlay approach. The only protein detected by both methods was the pilus biogenesis protein PilN. Neisserial PilN is a type IV pilus biogenesis

protein with orthologs in a multitude of Gram-negative species (Nudleman & Kaiser, 2004). The identification of the recombinase RecA (solid phase overlay assay) and the DNA helicase RecG (STM analysis) could be expected, since these proteins both are involved in recombination, which is the genome integration step of the transformation process (Sechman *et al.*, 2006). Furthermore, SSB was identified by our approach, while Tang and co-workers identified the homologous protein DprA. SSB and DprA are both listed as single-stranded DNA binding proteins in the neisserial genome annotation, and a functional relationship between orthologs of these proteins has been found in competence in *S. pneumoniae* (Mortier-Barriere *et al.*, 2007). Interestingly, Tang and co-workers observed a 100-fold reduction in the transformation rate of a Mc Δ*ptsI* mutant, suggesting that phosphorylation of PTS proteins might be relevant for neisserial transformation. This finding was previously reported in *H. influenzae* (Macfadyen *et al.*, 1996), indicating that competence might be regulated by the nutritional environment in both *Haemophilus* and neisserial species. Furthermore, the regulation of PTS proteins by phosphorylation has been suggested to play an important role in neisserial virulence (Deutscher *et al.*, 2005). Notably, the pilus biogenesis protein PilP was not detected in our solid phase overlay screening. PilP has exhibited evident DNA binding in EMSA analysis (Assalkhou *et al.*, unpublished observations). However, the negative PilP DNA binding in the solid phase overlay assay might represent one example of the limitations of this method, most probably due to incomplete renaturation of the lipoprotein on the blotting membrane. PilT, on the other hand, did not bind DNA at all in any of the DNA binding assays tested (Lång, Homberset, Alfsnes and Tønjum, unpublished data).

Table 4. Distribution of orthologs of the DNA binding proteins idenified in some major bacterial pathogens.

Protein	***N. meningitidis*** **MC58**	***P. aeruginosa*** **PAO1**	***H. influenzae*** **Rd KW20**	***S. pneumoniae*** **TIGR4**	***H. pylori*** **26695**
SSB NMB1460	+	+	+	+	+
RecA NMB1445	+	+	+	+	+
FtsE NMB0007	+	+	+	+	+
PilG NMB0333	+	+	+		
PilQ NMB1812	+	+	+		
ComL NMB0703	+	+	+		
PilM NMB1808	+	+			
PilN NMB1809	+	+			
PilO NMB1810	+	+			
PilC1 NMB1847	+	+			
NMB1796	+	+		+	
NMB0478	+				
NMB0086	+				
NMB1963	+				
Omp85 NMB0182	+	+	+		+
H.8 NMB1533	+	+			

4.4 Identification of orthologs of the DNA binding proteins identified

A homology search on the DNA binding proteins identified was performed on MicrobesOnline, using *N. meningitidis*, *P. aeruginosa*, *H. influenzae*, *S. pneumoniae* and *H. pylori* as selected genomes (Alm *et al.*, 2005) (Table 4). The term “homologs” to genes or proteins has been extensively used within science. Homology among proteins and DNA is often based on sequence similarity. In evolutionary biology, homology refers to any similarity between molecules that is due to their shared ancestry. Furthermore, there are three separate subgroups of homology: orthology, paralogy and xenology (Fitch, 2000). Orthologs are homologous sequences that were

generated in an evolutionary event, where one species formed two separate species. Interestingly, orthologs often, but not always, have the same function. Paralogs are homologous sequences that were separated by gene duplication. Finally, homologs resulting from horizontal gene transfer between two organisms are termed xenologs (Fitch, 2000). In this Thesis I use the term "orthologs" instead of "homologs", since orthology constitutes a more precise description of the relatedness between proteins identified. Interestingly, our findings suggest a conservation of SSB, RecA and FtsE among all bacterial species. In *B. subtilis* two paralogous genes encode for SSB proteins, one essential for cell survival and one required for transformation. In fact, most of the naturally transformable bacteria harbor multiple *ssb* genes within their genomes, with the exception of *H. pylori* and *Campylobacter jejuni* (Lindner *et al.*, 2004). Furthermore, orthologs of PilG are widely distributed among Gram-positive and Gram-negative organisms (**Paper III**) (Collins *et al.*, 2007), while orthologs of PilQ and ComL are both widely distributed among Gram-negative bacterial species (Malinverni *et al.*, 2006; Martin *et al.*, 1993). Orthologs of the neisserial pilus biogenesis components PilM, PilN, PilO and PilC1 were also found in *P. aeruginosa*, which hosts an abundance of type II secretion pathways (Ball *et al.*, 2002; Durand *et al.*, 2003). Furthermore, only one of the hypothetical components identified, NMB1796, has orthologs in other species than *Neisseria*, indicating that the other hypothetical proteins identified might be important in neisserial pathogenicity. Moreover, the outer membrane proteins H.8 and Omp85 show different distribution patterns.

4.5 The role of pilus retraction in pilus biogenesis and neisserial pathogenesis

Type IV pilus biogenesis constitutes a dynamic process of rapid polymerization and depolymerization of pilin subunits, also mediating twitching motility during pilus retraction (Merz & So, 2000; Wolfgang *et al.*, 2000). Pilus expression is important for the initial adherence of neisserial species to epithelial and endothelial cells (Heckels, 1989; Pujol *et al.*, 1999; Stephens & McGee, 1981), while pilus retraction mediates an intimate contact between the bacterium and the host cell. Subsequently, opacity proteins and adhesins establish a close contact to the host cell membrane, initiating the infection (Dehio *et al.*, 1998; Nassif *et al.*, 1999). Thus, pilus biogenesis as well as the balanced regulation of pilus stability is important in neisserial pathogenicity (Carbonnelle *et al.*, 2006; Tønjum & Koomey, 1997).

4.6 On the role of PilG in transformation

The inner membrane component PilG is required for pilus biogenesis, since Δ*pilG* null mutants are non-piliated (Tønjum *et al.*, 1995). Interestingly, PilG was found to bind DNA in our solid phase overlay assay. PilG bound DNA in a DUS non-specific manner and no preference for single-stranded or double-stranded DNA was observed (**Paper I**). DNA interaction studies of PilG partial proteins and subsequent endoproteinase cleavage of these proteins revealed the importance of residues 1 to 30 and 60 to 80 in PilG DNA binding (Fig. 6). These findings were verified by EMSA analysis (**Paper III**).

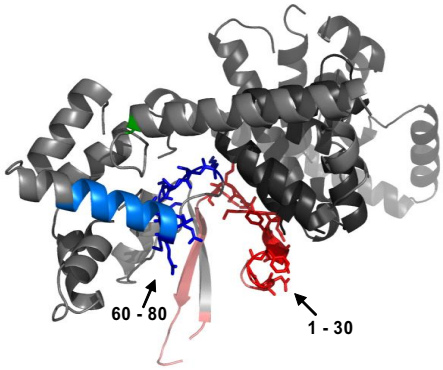

Fig. 6. 3D structure prediction of the *N. meningitidis* (Mc) PilG protein. Regions 1 to 30 are shown in red/pink and regions 60 to 80 are shown in blue/dark blue. According to this structure, the two regions of interest are predicted to be in close proximity to each other.

In addition, PilG directly interacts with the outer membrane secretin complex PilQ, based on the results from Far-western analysis that N-terminal PilQ binds N-terminal PilG. The finding that N-terminal PilQ interacts with PilG is consistent with previous findings on PilQ topology, showing that the N-terminus of PilQ is directed towards the inner membrane (Frye *et al.*, 2006). Furthermore, PilG also interacts with full-length recombinant ComL (**Paper III**). The fact that the N-terminus of PilG interacts with both DNA and proteins indicates that PilG exerts dual functions. While residues 1 to 30 and 60 to 80 appear to be important for DNA binding, the more C-terminal part of the N-terminus might be able to contribute to the protein-protein interactions observed.

In order to enlighten the function of PilG DNA binding, a Δ*pilG* null mutant was constructed and shown to be defective in pilus biogenesis and transformation (**Paper I**). Yet, the interpretation of the biological significance of the DNA binding capabilities of PilG is complicated by the fact that it participates in type IV pilus biogenesis, which is required for competence. Thus,

it is a conundrum whether the lack of competence in this mutant is due to a defect in the direct binding of DNA, or whether it is indirect, through pilus biogenesis.

As indicated by recent PilG structural predictions (Collins *et al.*, 2007), as well as our own bioinformatics predictions (**Paper III**), the N-terminus of PilG is located in the cytoplasm. DNA binding activity in this part of the protein might indicate that PilG contributes to the entry of exogenous DNA into the neisserial cell, or act as an intermediate chaperone in the vicinity of the inner membrane. Thus, there is a possibility that PilG might function as a transport protein, in close contact with the ComA pore.

Recently, the Δ*pilG*/*pilT* double mutant was shown to exhibit restored pilus expression, in addition to pilus-associated adherence and aggregation properties (Carbonnelle *et al.*, 2006). Thus, PilG was suggested to play a role in regulating pilus homeostasis by counteracting the retractive function of PilT. This type of functional relationship was previously observed between the pilus adhesin PilC proteins and PilT (Morand *et al.*, 2004). In contrast, the PilG ortholog BfpE interacts with the PilT-like ATPase BfpF, promoting retraction of bundle-forming pili (BFP) in enteropathogenic *E. coli* (EPEC) (Crowther *et al.*, 2004). In addition, physical protein-protein interactions between PilG and PilT orthologs have been observed in *P. aeruginosa* and *Erwinia chrysanthemi* (Arts *et al.*, 2007; Py *et al.*, 2001). In our hands, Far-western analysis indicated no direct interaction between PilG and the putative ATPases PilT and PilF (**Paper III**) (Wolfgang *et al.*, 1998). The contradictions seen in these functional studies on PilG may be due to the structural differences that exist between PilG orthologs. For instance, XcpS is predicted to have three transmembrane helices, while four predicted transmembrane helices were identified in PilG and BfpE (Arts *et al.*, 2007; Blank & Donnenberg, 2001; Collins *et al.*, 2007). Furthermore, neisserial PilG has a tendency to form tetramers and there is evidence for multimerization of BfpE (Collins

et al., 2007; Crowther *et al.*, 2004). Consequently, according to published 3D structures of the PilQ complex and the PilG multimer, the PilQ dodecamer can be perceived as a tetramer of trimers (Collins *et al.*, 2004), with one PilG molecule per PilQ trimer (Collins *et al.*, 2007). Our bioinformatics predictions corroborate the existence of three transmembrane helices in the PilG structure (**Paper III**). PilG multimerization studies constitute ongoing work in our group, in order to confirm or revise the published tetrameric structure of PilG (Collins *et al.*, 2007). In addition, crystallization trials of N-terminal PilG are currently in progress.

Studies on PilG self-assembly and interactions with other membrane proteins, will contribute to improved understanding of the function of PilG. Although structural and functional studies on PilG and its orthologs are contradictory, the importance of PilG in pilus biogenesis and transformation is broadly recognized. Future studies will help in revealing the exact role of PilG in transformation and the biological significance of its DNA binding activity.

4.7 Methodological considerations

4.7.1 Isolation of proteins from neisserial cellular fractions

Important aspects of neisserial membrane fractionation were previously documented (Masson & Holbein, 1983; Pannekoek *et al.*, 1992). Fractionation studies on *E. coli* demonstrated Sarkosyl-specific solubilization of inner membrane (IM) proteins, in comparison to Triton X-100 (Filip *et al.*, 1973). In the process of optimizing neisserial membrane fractionation, Sarkosyl was shown to be the optimal detergent for IM protein isolation (**Paper I**). However, due to published constrains in neisserial membrane separation (Masson & Holbein, 1983), complete separation of the

membranes was not achieved by this method. Noteworthy, we obtained protein separation through ultracentrifugation, while Filip and co-workers employed sucrose density gradient centrifugation (Filip *et al.*, 1973). Sucrose density gradient centrifugation was developed in membrane separation studies in *E. coli* (Schnaitman, 1970) and *Salmonella typhimurium* (Osborn *et al.*, 1972), and this method separates components according to sedimentation speed, while ultracentrifugation separates soluble and insoluble components. The cytoplasmic protein SSB was the single predominant DNA binding protein reproducibly detected in the neisserial soluble fractions, indicating that the method employed either failed to provide enrichment of periplasmic proteins or that the DNA binding proteins in that fraction were not expressed at high enough levels to be detected by the solid phase overlay assay (Ames *et al.*, 1984; Judd & Porcella, 1993). Chloroform treatment apparently cause disruption of both cellular membranes, releasing all soluble proteins of the neisserial cell. Furthermore, this result also suggests that substantial protein denaturation and/or degradation might have occurred. Importantly, the action of chloroform in this assay has not been documented (**Paper II**).

4.7.2 1D versus 2D electrophoresis

The advantage of a conventional two-dimensional (2D) electrophoresis system is the improved resolution of membrane-associated components and components with low hydrophobicity, as compared to 1D. More complete separation of proteins with the same or similar molecular mass is achieved by the isoelectric focusing (IEF) in the first dimension (**Paper II**). However, membrane proteins with high hydrophobicity are not readily separated by conventional 2D and additional techniques, developed to comprehend with this problem, are available (Braun *et al.*, 2007).

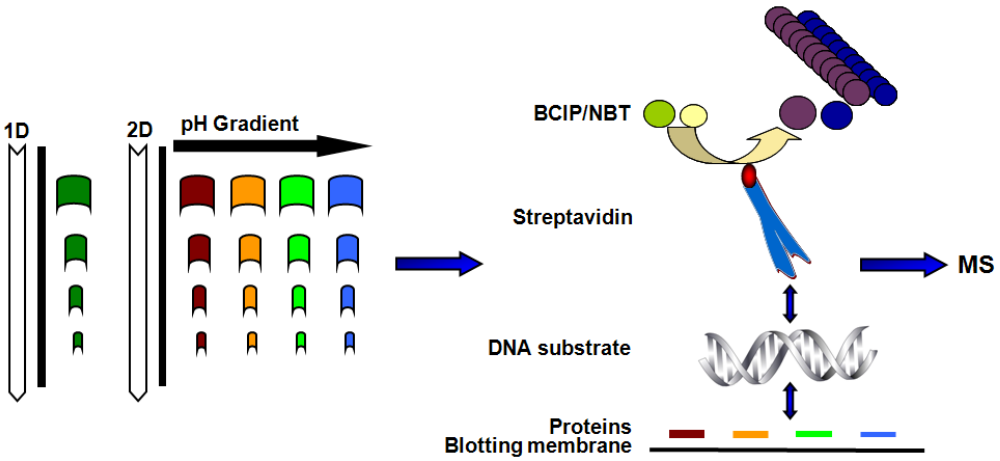

Fig. 7. Procedure for solid phase overlay assay with DNA substrates in combination with 1D/2D gel electrophoresis and mass spectrometry (MS) analysis.

4.7.3 Searching for DNA binding components

The solid phase overlay assay in the form of South-western analysis was first described in 1980 (Bowen *et al.*, 1980) (Fig. 7). Even though this method provides reproducible results, it has limitations such as limited sensitivity, detecting only proteins that are expressed at a certain level, and dependence on correct protein folding. A main limitation is the inability of some proteins to renature after SDS-PAGE. Consequently, some DNA binding candidates were not detected by this assay, and in some gel fractions so many proteins were present that reproducibility was reduced (**Papers I and II**). Therefore the experiments must be supplemented by other methods. Furthermore, quantification of the affinity of the DNA binding activity is not available with this method, but would require monitoring of protein-DNA interactions by for example plasmon resonance analysis. Yet, the reproducible identification of PilG, ComL and PilQ DNA binding by South-western analysis, in addition to other independent assays (Assalkhou *et al.*, 2007) (**Papers I-III**), is strong evidence that the method is indeed valid for a number of proteins. In combination

with 2D electrophoresis, the approach employed is a good tool for screening complex mixtures of proteins and identifying candidates with DNA binding activity (**Papers I and II**).

In comparison, EMSA is a quantitative, non-denaturating method, used for assessing the DNA binding activity of single components. Since proteins are kept in their native form, this method is considered more valid (**Papers II and III**).

Importantly, DNA binding activity observed *in vitro* may or may not be functional *in vivo* or it might be relevant in another biological process than the one under study. Thus, the identification of DNA binding proteins not related to transformation does not exclude a biological significance of the DNA binding activity detected (**Paper I**).

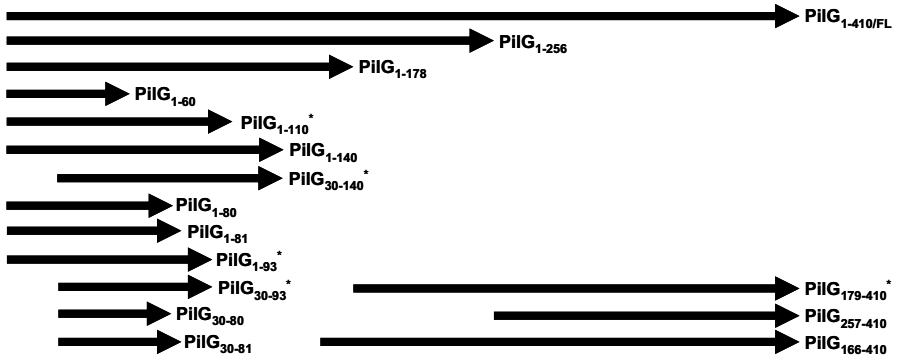

Fig. 8. Schematic overview of all PilG constructs made in this study. *

4.7.4 Over-expression and purification of PilG partial proteins

Membrane protein assembly is a complex process, however, the "positive–inside" rule has revealed some insight into transmembrane topology of integral membrane proteins (von Heijne, 1989; von Heijne, 2006). Among the challenging tasks in over-expression and purification of

* PilG partial constructs which did not yield expressed protein.

membrane proteins are retaining protein stability and function, in addition to providing conditions for protein solubility versus cytoplasmic aggregation and degradation (Drew *et al.*, 2003). The constructs encoding $PilG_{1-93}$, $PilG_{30-93}$, $PilG_{1-110}$, $PilG_{30-140}$ and $PilG_{179-410}$ did not yield expressed protein (Fig. 8). Since the smaller partial proteins were all easily expressed and soluble, these longer proteins might be unstable. Furthermore, the different expression patterns obtained with $PilG_{166-410}$ and $PilG_{179-410}$ indicate that a short cytoplasmic tail is required to achieve a stable and expressible protein.

Another interesting aspect is the requirement for n-Dodecyl β-Maltoside (DDM) to purify soluble proteins of $PilG_{1-140}$ and $PilG_{1-178}$, proteins expected to be soluble due to lack of transmembrane helices. Since this phenomenon was not observed among the smallest partial proteins, the longer partial proteins might contain regions which promote self-aggregation. Previous studies have documented the use of DDM in protein solubilization (Lund *et al.*, 1989; VanAken *et al.*, 1986). Furthermore, proteins $PilG_{30-80}$ and $PilG_{30-81}$ accumulated in inclusion bodies and could only be solubilized in urea, suggesting that the first residues of the N-terminus are important in protein solubility (**Paper III**).

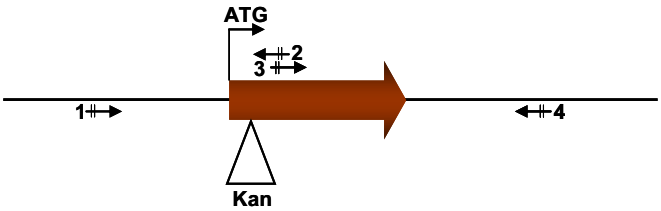

Fig. 9. Schematic model of the construction of null mutants by the insertion of a kanamycin resistance gene cassette in the start region of the gene of interest.

4.7.5 Null mutant construction and testing for competence

Null mutants of DNA binding components identified were constructed by the insertion of a kanamycin resistance gene in the start codon of each target gene (Fig. 9). Three null mutants were not viable, Δ*comL*, Δ*omp85* and Δ*ssb*, thus their phenotypes associated with competence could not be assessed in these strains (Table 3) (**Papers I and II**). Noteworthy, Fussenegger and co-workers were able to generate a viable Δ*comL* mutant in *N. gonorrhoeae* through transposon shuttle mutagenesis (Fussenegger *et al.*, 1996a), where a transposon insertion was located at amino acid 112. This Δ*comL* mutant exhibited a reduced transformation rate as compared to wildtype. Thus, the N-terminus of ComL seems to be important for protein expression and function. Omp85 is an essential OM protein involved in the folding and assembly of OMPs into the outer membrane (Knowles *et al.*, 2009). Conditional mutants of *omp85* have been constructed, however, the mutants exhibited growth defects (Genevrois *et al.*, 2003; Voulhoux *et al.*, 2003). Moreover, mutant construction of the C-terminus of the *E. coli* ortholog of *ssb* has emphasized the importance of SSB in protein interactions and survival (Shereda *et al.*, 2008).

Competence for transformation was assessed by using the plasmid pSY6 as donor DNA. pSY6 contains a point-mutated copy of a DNA gyrase gene, conferring resistance to nalidixic acid when incorporated into the chromosome (Stein *et al.*, 1991). The construction of null mutants was performed in order to test the significance of the proteins identified in relation to competence for transformation. Since type IV pilus expression is a requirement for competence, mutant construction and competence testing of type IV pilus biogenesis components also requires the construction of inducible constructs (**Papers I and II**). Mutants that did not have a phenotype in competence, might be relevant in other aspects of nerisserial DNA metabolism.

4.7.6 Additional approaches for assessing protein interactions

Once protein-DNA and protein-protein interactions are detected *in vitro*, their reproducibility should be tested by alternative methods and their biological significance should be challenged. Such interactions may differ in their nature by ranging from stable to transient, influencing the method required for their detection. Protein-DNA and protein-protein interactions relevant for the process under study may have low affinity and/or short half lives. Therefore, one may have experimental difficulties in detecting these interactions and underestimate the quantity of interacting complexes. One challenge is thus to try to preserve as much as possible, also of labile complexes, with the risk of attaining false positive reactions. There are multiple ways to detect protein-protein interactions in addition to Far-western analysis, for example by the yeast two hybrid (Y2H) system, peptide arrays, protein microarrays, cross-linking and co-immunoprecipitation (Co-IP). Furthermore, the strength of the protein interactions can be estimated by surface plasmon resonance analysis, monitoring the affitnity of interacting proteins in real time (Jason-Moller *et al.*, 2006).

Currently, laser tweezer traps constitute an attractive approach for the visualization and measurement of dynamic forces of molecular machines (Allemand & Maier, 2009; Fällman *et al.*, 2004). A local laser tweezer trap facility will be employed to delineate the forces that enable pilus retraction in *Neisseria*, most importantly the functional relationship between PilG and PilT, and to visualize the potential relationship of DNA to the pilus during pilus retraction. Furthermore, *in situ* analysis of all components involved in neisserial transformation including PilG, ComL, SSB and RecA will reveal new insights into the functional relationships between these proteins.

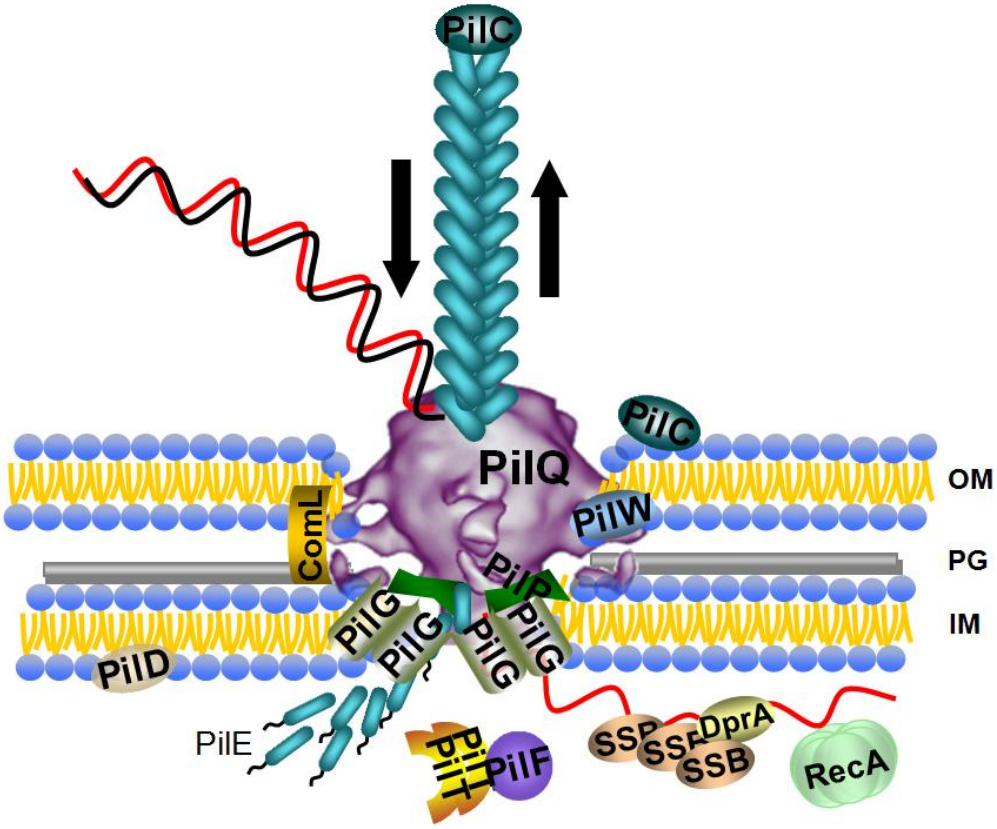

Fig. 10. Proposed model of the neisserial transformation machinery. A number of previously recognized pilus biogenesis components in combination with novel findings are depicted. OM = Outer membrane, IM = Inner membrane, PG = Peptidoglycan layer

5. Model of the neisserial transformasome

Combining the findings presented with already established models enabled a modified view of neisserial DNA uptake with the main focus on the functional relationships between PilQ, PilG and ComL (Fig. 10). Based on our previous findings (Ambur *et al.*, 2009; Balasingham *et al.*, 2007; Davidsen & Tønjum, 2006), and result on the enteropathogenic *E. coli* (Hwang *et al.*, 2003), we propose that a protein complex spanning the outer (OM) and inner (IM) membranes, periplasm and peptidoglycan layer form the neisserial transformasome. We have provided evidence that PilG directly interacts with both PilQ and ComL, which corroborates the previously documented PilQ-PilP interacting complex (Balasingham *et al.*, 2007). In our model, we suggest that DNA is bound at the Mc cellular surface and taken up and transported to the IM through the PilQ complex. Subsequently, ComL and PilG are predicted to contribute to the entry of DNA into the cytoplasm. Finally, SSB binds to the incoming single-stranded DNA, delivers it to RecA and DNA can be introduced into the genome by homologous recombination. So far, the components exerting nuclease activity and DUS specificity have not been identified. The DNA binding properties of PilG and ComL observed, in addition to their direct interaction, might suggest that these proteins have synergistic functions during transformation. As previously proposed, ComL is closely associated with the OM and BAM proteins (Knowles *et al.*, 2009) and might open up the peptidoglycan layer (Fussenegger *et al.*, 1996a), mediating transfer of DNA from PilQ. The DNA binding properties of ComL support a previous report on a potential role for this protein in neisserial transformation and indicate that the interaction with DNA is of a direct nature. Furthermore, the true function of ComL is difficult to elucidate due to its essential character, however, the assessment of its precise cellular location will help elucidate the potential role of ComL in transformation. In the IM, PilG might play a role as a chaperone or transport protein.

However, since Δ*pilG* null mutants are non-piliated (Tønjum *et al.*, 1995), PilG is suggested to also play a role in the assembly of pilin subunits into a pilus rod and/or can be of importance in the stabilization of polymerized pili. In addition, PilG could be involved in DNA processing. This would explain the dual function observed for the N-terminus of PilG in exhibiting both DNA binding activity and direct binding of PilQ and ComL.

6. Future perspectives

In order to improve strategies for prevention and treatment of meningococcal infection and disease, in addition to generate new drugs and vaccines, it is vital to understand the basis of neisserial DNA metabolism. Studies on DNA uptake in pathogens and its relationship to genome instability and strain variation have been broadly documented. The balance between these processes determines the outcome of genome variability, bacterial fitness for survival and virulence. In addition, neisserial transformation has been suggested to be linked to DNA repair (Davidsen *et al.*, 2004).

Although a lot of questions remain, the findings presented have changed the current view of the transformation pathway in *Neisseria*. The observation that PilG binds DNA and also interacts with multiple proteins involved in type IV pilus biogenesis and transformation raise questions on the biological role of PilG. The lack of evidence of direct physical interaction between PilG and the ATPase PilT is puzzling. However, a functional yet indirect interaction between these components might exist, as previously suggested in several bacterial species, although findings are contradictory (Arts *et al.*, 2007; Carbonnelle *et al.*, 2006; Crowther *et al.*, 2004; Py *et al.*, 2001). Thus, in order to fine-tune our model of the neisserial transformasome, it would be interesting to assess the functional relationship between PilG and PilT and other components in neisserial strains.

Ever since DUS was found to mediate strain-specific DNA uptake (Goodman & Scocca, 1988), questions on how the selection between foreign and self/homologous DNA and DUS recognition takes place and what component(s) is involved, have lingered in the minds of many scientists. Even though transformation contributes to genetic diversity, DUS-mediated transformation has been shown to mediate genome conservation (Treangen *et al.*, 2008), thereby

transformation also plays a role in genome maintenance (Davidsen *et al.*, 2004). Our search for DNA binding components did, however, not enlighten this particular challenge and no DUS-specific receptor is yet identified. Hopefully, upcoming research focusing on the neisserial DNA transformation pathway will solve this mystery.

In order to acquire an improved understanding of the neisserial transformation pathway, the following questions should be addressed:

- What are the functional relationships between PilG, PilQ, ComL and PilT and what is the role of the DNA binding property of PilG and ComL in these interactions?
- What are the exact amino acid residues mediating the PilG DNA and protein interactions observed?
- What is the main function of PilG and which proteins does it interact with?
- What is the role of ComL in transformation and which part of ComL interacts with PilG and DNA?

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